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Pythium abappressorium—a new species from eastern Washington

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Abstract: A new species of *Pythium* isolated from wheat and apple roots in eastern Washington is described. *Pythium abappressorium* sp. nov. is characterized by abundant appressoria. Plerotic oospores and sporangia are formed from the appressoria and remnants of the appressoria remain attached to the base of sporangia at maturity. Smaller appressorial swellings, reminiscent of hyphal swellings, are also formed within the appressoria. *Pythium abappressorium* is pathogenic to wheat, causing damping-off and stunting, but is not pathogenic to apples. The fungus can grow in the temperature range 5 to 30 C, with an optimum of 20 C. The sequence of the ITS1 region of the rDNA did not match the sequences from a worldwide collection of over 1200 isolates, including types and neotypes, suggesting that this species has not been previously described.

Key Words: apple, appressoria, biological control agent, damping-off, *Malus domestica*, root rot, *Triticum aestivum*, wheat

INTRODUCTION

Over two million acres of dryland wheat and barley are grown in eastern Washington. These areas are characterized by deep wind-deposited loess soils, and range in rainfall from 22 to 65 cm per year. The most easterly part of Washington was formerly a grassland prairie on rolling hills. As of the late 1980s, 19 species of *Pythium* had been reported to be pathogenic on wheat and 10 on barley in North America (Farr et al 1989). Pythium root rot has been demonstrated to stunt plants and reduce yields in the Pacific Northwest, based on plots treated with metalaxyl, which is specific for Oomycetes (Cook et al 1987). Chamswarng and Cook (1985) isolated ten species of *Pythium* and two unidentified *Pythium* isolates from fields around Pullman, Washington and Moscow, Idaho, and showed that all were pathogenic. Despite the importance of *Pythium* in cereal production, little is known about which species are predominant, both in terms of populations and pathogenicity, across this or any wheat-growing region. *Pythium* spp. are difficult to key out, especially heterothallic isolates or those that are completely asexual, because these species do not form sexual structures in culture that are required for identification. However, recent molecular techniques such as PCR and sequencing may be useful for ascertaining the genetic diversity of *Pythium* spp. in wheat and barley. In the summer of 2000, over 80 sites across Eastern Washington and Northern Idaho were sampled for *Pythium* spp, as part of a larger project to describe the species and population diversity of this fungal genus. The ITS 1 region of the rDNA was amplified by PCR and sequenced for a subsample of this collection, and compared to a sequence database of type species from a worldwide collection (C. A. Lévesque, unpubl). One set of isolates represented a taxon widely distributed in most sites and lacking sequence homology and morphological similarity to known species in the keys of van der Plaats-Niterink (1981) or Dick (1990). These isolates also resembled unknown isolates found by Mazzola et al (2002) in apple orchards in the Wenatchee area of central Washington. These latter isolates were recovered from the roots of apple at three of six orchards surveyed in central Washington. All isolates of this undescribed species exhibited moderate to high levels of resistance to the fungicide metalaxyl regardless of orchard source. Application of certain isolates to roots stimulated growth of apple in sterile soil and reduced root infection when seedlings were planted in soil infested with pathogenic *Pythium* spp. including *P. ultimum* Trow and *P. sylvaticum* Campbell & Hendrix. Based on the unique morphological characteristics and ITS sequence, a new species is described.

MATERIALS AND METHODS

Isolate collection and maintenance.—In July and August, 2000, soil and root samples were collected from wheat and

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barley fields in Adams, Columbia, Garfield, Lincoln, Spokane, Walla Walla, and Whitman counties of Eastern Washington. Samples were kept at 4 C until isolates were processed for recovery of *Pythium*. Three 10-g samples containing both roots and soils were placed in 15-cm petri dishes and rewetted with 2–3 mL of water (approximate field capacity) and incubated at 22 C for 24–48 h. Plates were then flooded with distilled water, and grass clippings (*Poa pratensis* L.) were floated on the water. After 24–36 h, the grass clippings were plated on *Pythium* selective medium (Mircetich and Kraft 1973), made up in water agar instead of corn meal agar. One day later, hyphal tips were removed using a pasteur pipette drawn out to a fine tip, and plated on water agar. Cultures were also placed on PDA and V8 agar, and grown on autoclaved grass clippings in sterile pond water for identifications. Appressoria were observed on cellophane membranes overlaying cultures on corn meal agar. Cultures were stored at 4 C on PDA slants and sterile oat seeds. Morphological characters were compared to the keys and descriptions of van der Plaats-Niterinck (1981), Dick (1990), Matthews (1931), Middleton (1943), Waterhouse (1968), and more recent species descriptions from the original papers cited in Index of the Fungi, Vol. 6 and Dick (2000).

Pathogenicity testing.—Inoculum of *P. abappressorium* was prepared in autoclaved sandy loam amended with 1% ground rolled oats (Paulitz and Baker 1987). The oatmeal-soil was prepared in 1-quart narrow mouth Mason jars, with a 0.5-cm hole drilled in the lid, with a 70-mm diameter filter disk (Fungi Perfecti, Corvallis, Oregon) placed inside the lid to maintain air exchange and sterility. Each of two strains (010111 and 010112) was separately transferred to the jars in the form of PDA plugs. The jars were shaken, and incubated for 3 wk. Propagule density was assessed with dilution plating on Pythium selective medium. Inoculum was mixed by hand into pasteurized sandy loam (from WSU Dryland Research Station, Lind, Washington) at a rate of 1000 cfu/g and placed in 10-cm square pots. Controls consisted of pasteurized sandy loam without any inoculum added. Four replicate pots were used for each treatment, and each pot was planted with five seeds of *Triticum aestivum* L. cv. Penawawa. Pots were placed in a temperature controlled growth room at 16 C, 12 h light/dark.

Effect of temperature on growth.—The growth rate of isolates 020125, 020135 and 90089 were measured on 15-cm-diameter petri plates of PDA. Three replicate plates of each isolate were inoculated with a 3-mm plug, and placed in a growth chamber at 5, 10, 15, 20, 25, 30, and 35 C. One experiment was done separately for each temperature.

DNA extraction, PCR amplification, and sequencing.—DNA was isolated from 7- to 10-d-old cultures of isolates grown in 5 mL of potato dextrose broth (Difco, Becton Dickinson) in 12.5 cm × 1.5 cm tubes at room temperature on an orbital shaker at ca 110 rpm. Mycelial mats were washed 1× with distilled water then added to FastDNA tubes (FastDNA Kit, Bio101, Carlsbad, California 92008) with 200 μL sterile distilled water. One mL of CLS-Y cell lysis solution was added and samples were homogenized in an FP 120 FastPrep Cell Disruptor (Thermo Savant, Holbrook, New York 11741) at speed 4 for 40 s. Samples were then centrifuged at 12 000 rpm for 1 min and the supernatant was removed to a clean 1.5 mL microcentrifuge tube. Six-hundred μL of binding matrix was added, and tubes mixed by inversion and incubated at room temperature for 5 min. The tubes were then centrifuged at 12 000 rpm for 1 min and the supernatant was discarded.

Pellets were resuspended in 500 μL SEWS-M by stirring with a pipette tip then centrifuging at 13 000 rpm for 1 min. After removal of the wash solution, tubes were spun 5 s and residual solution was pipetted off before eluting DNA. Matrix was resuspended in 100 μL DES by stirring with a pipette tip followed by incubation at room temperature for 3 min. Samples were then centrifuged at 12 000 rpm for 1 min and the supernatant was removed to a 0.5 mL microcentrifuge tube.

ITS PCR.—DNA was amplified with ITS1 or ITS2 region primers. ITS1 primers: UN-UP18542, 5′ cgtaacaaggtttccgtaggtgaac 3′and OOM-LO5.8S47B, 5′ cgcattacgtatcgcagttcgcag 3′. ITS2 primers: OOM-UP5.8S01, 5′ caactttcagcagtggatgtct 3′ and PY-LO28S22, 5′ gtttcttttcctccgcttattaatatg 3′ (Lévesque et al 1998). Taq polymerase, 10× reaction buffer, and magnesium were obtained from Promega (Madison, Wisconsin 53711). The 25-μL reaction mixture contained 1.5 mM magnesium, 10 pmoles of each primer, 200 uM dNTPs, and 1 Unit Taq polymerase. The cycling conditions for ITS1 primers were 94 C for 2 min, 32 cycles of 94 C for 45 s, 60 C for 45 s, 72 C for 1 min, followed by 1 cycle of 72 C for 10 min. Cycling conditions were the same for the ITS2 primers with the exception of an annealing temperature of 52 C.

Sequencing of ITS PCR products.—Five μL of each PCR product was treated by incubating with 2 uL ExoSAP-IT (United States Biochemical, Cleveland, Ohio 44121) for 30 min at 37 C followed by 15 min at 80 C. Sequencing reactions contained 5 μL of ExoSAP-IT treated PCR product, 4 μL Big Dye Mix (ABI-Prism, Foster City, California 94404), 1 μL of 3.2 pmoles/μL primer. An MJReserch PT-200 thermocycler was used to run reactions with the following cycling parameters: 2 min at 94 C, 25 cycles of 1 min at 94 C, 1 min at 50 C, and 1 min at 60 C followed by holding at 4 C. After cycling, 10 μL of distilled water was added to each sequencing reaction. Each diluted reaction was cleaned over a Sephadex G-50 fine mini spin column, dried in a speed-vac, resuspended in 4 μL of loading dye, and 2 μL of the reaction/dye mix was run on an ABI 377 sequencer.

TAXONOMY

Pythium abappressorium T. C. Paulitz et M. Mazzola, sp. nov. FIGS. 1–8

Coloniae in agaro PDA plerumque ordinatione indistincta radiante, hyphis usque ad 5 μm crassis compositae, cum appressoriis multis usque ad 160 μm longis, 8–12 μm crassis efferentes. Appressoria curva vel falcate saepe ramose vel catenata, ad connectivum constricta. Zoosporangia plus minusve globosa, (11–)16–22(–30) μm, terminalia vel in-

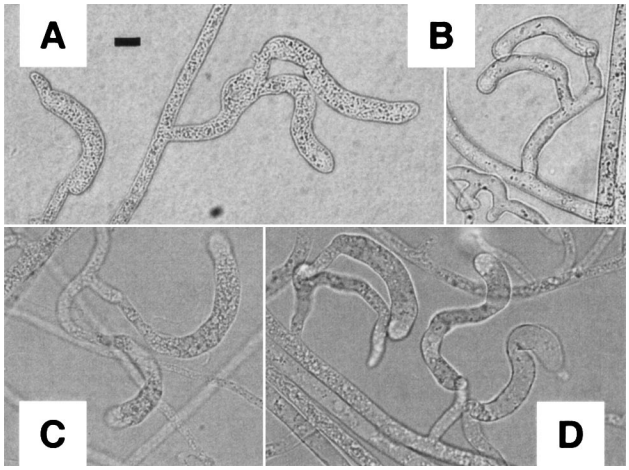

FIG. 1. Appressoria of *P. abappressorium* formed on cellophane membranes on corn meal agar plates. Appressoria are sickle- (FIG. 1B, C) or S-shaped (FIG. 1D), with 2 or more appressoria arising from a common stalk. Appressoria are constricted at point of attachment with stalk. Bar represents 10 μm.

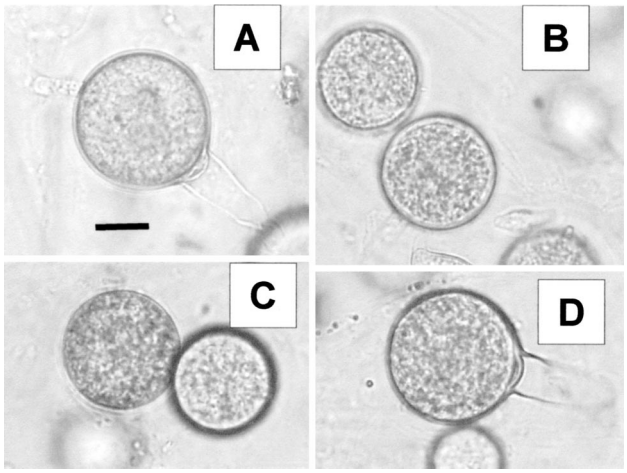

FIG. 3. Sporangia of *Pythium abappressorium*. Sporangia formed intercalary or terminal from hyphae or appressoria. Tapered remnants of appressoria often remain attached to base of sporangium (A, D). Bar represents 10 μm.

tercalaria vel in appressoriis efferentia; reliquiis appressorii saepe affixas ad basim sporangii. Zoosporae ad 20 C in zoosporangiis formantia; tubis 2–4 μm longis emittens. Tumores appressoriorum et hypharum globosi vel limoniformes vel cylindrici, (11–)13–22(–24) μm longi, (8–)10–18(–20) μm crassi, in hyphis terminalis vel intercalaris vel appressoriis efferentes; septa saepe tumors appressoriorum ex appressoriis evacuatis separata. Oogonia levia, terminalia vel in appressoriis vel in hyphis efferentia. Antheridia sacccata vel collo flexo, 7–15 μm longa, 4–9 μm crassa, plerumque monoclina, interdum hypogena, 1–3 per oogonium. Oosporae globosae leviae, plerumque pleroticae, (12–)14–17(–27) μm diam, parietis 1–2 μm crasso, 1 vel interdum 2 per oogonium efferentes.

Colonies on PDA usually with a vague radiate pattern, composed of hyphae up to 5 μm wide, producing many appressoria up to 160 μm long, 8–12 μm wide (FIGS. 1, 2). Appressoria curved to sickle-shaped (FIG. 1), often branched (FIG. 2) or in chains, constricted at point of connection. Zoosporangia more or less globose, (FIG. 3), (11–)16–22(–30) μm, terminal or intercalary or formed from appressoria; remains of appressorium often attached to the base of zoosporangium (FIG. 3A, D). Zoospores forming at 20 C in zoosporangia, discharging by way of exit tubes 2–4 μm long (FIG. 4). Appressorial and hyphal swellings globose, lemon-shaped, or cylindrical, terminal or intercalary in hyphae or appressorium (FIG. 5), (11–)13–22(–24) μm long, (8–)10–18(–20) μm wide, with septa often separating appressorial swell-

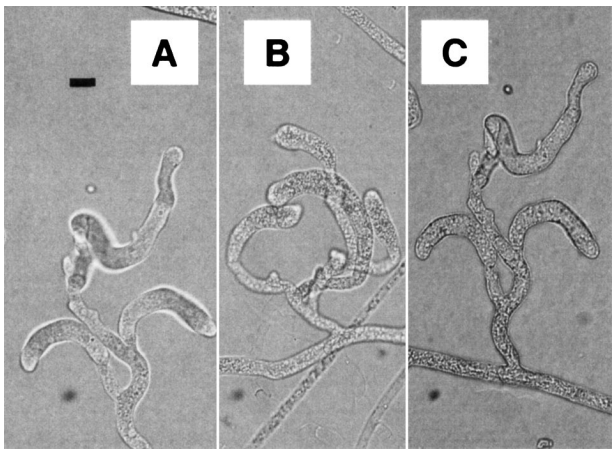

FIG. 2. Branched appressoria of *P. abappressorium* formed on cellophane membranes on corn meal agar plates. Bar represents 10 μm.

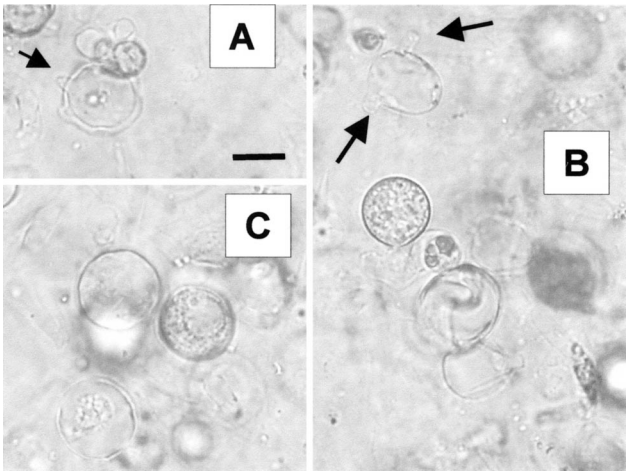

FIG. 4. Empty sporangia of *Pythium abappressorium*, after release of zoospores. Exit tubes are visible in A and B (arrows). Bar represents 10 μm.

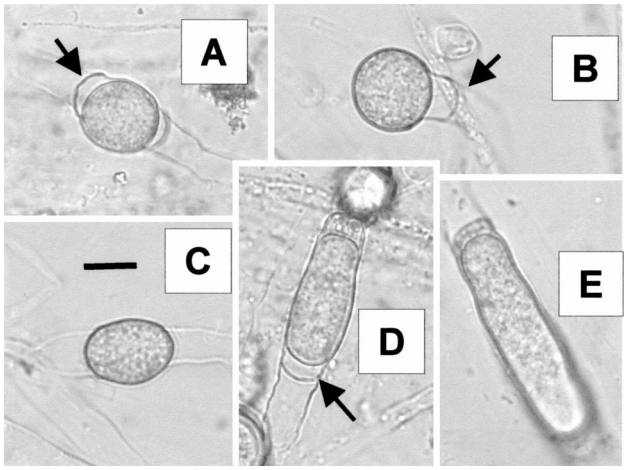

FIG. 5. Appressorial swellings of *Pythium abappressorium*. Swellings are mostly intercalary, sometimes terminal (B), globose, limonoform, or cylindrical (D, E). Septa often separating appressorial swelling from empty appressoria (arrows). Bar represents 10 μm.

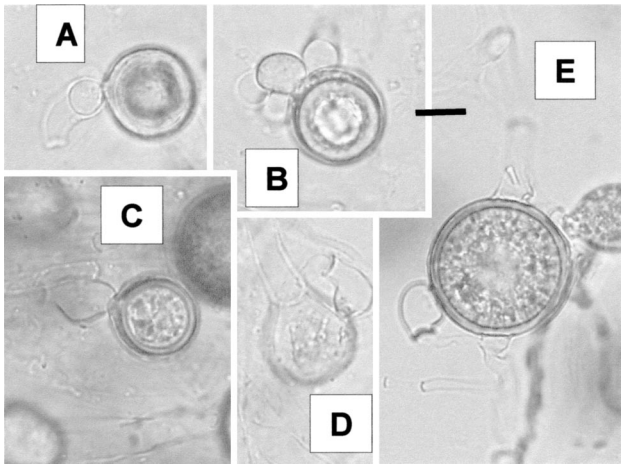

FIG. 7. Oogonia (A, B, D), oospores (C, E) and antheridia of *Pythium abappressorium*. Antheridia are globose, swollen and often curved (A, D, E), and usually monoclinous (D, E). Usually one antheridium per oogonium, but occasionally 2–3 (B). Bar represents 10 μm.

ings from empty appressorium (FIG. 5A, B, D). Oogonia smooth, terminal or produced within hyphae or appressoria (FIG. 6). Antheridia sac-shaped to crook-necked (FIG. 7), 7–15 μm long, 4–9 μm wide, mostly monoclinous, occasionally hypogenous (FIG. 8A, B), 1–3 per oogonium. Oospores smooth, globose, usually plerotic (FIG. 8), (12–)14–17(–27) μm diam with wall 1–2 μm thick, 1 or occasionally 2 per oogonium (FIG. 8C).

Daily growth rate on PDA– 3 mm at 5 C, 7 mm at 10 C, 13 mm at 15 C, 17 mm at 20 C, 15 mm at 25 C, 15 mm at 30 C and no growth at 35 C. Both isolates of *P. abappressorium* caused damping-off of wheat, and reduced emergence from 95% to 10%. In addition, seedlings that emerged were stunted, and the first true leaf was stunted and curled around, symptoms typical of embryo infection by *Pythium* in the seed (Hering et al 1987). The nucleotide sequence of the ITS1 region and part of the 5.8S ribosomal gene of *P. abappressorium* (366 bases) has been submitted to GenBank (AY082670).

Specimens examined. UNITED STATES, WASHINGTON: Pullman, Whitman Co, Lat: 46° 45′ 19″ N, Long. 117° 04′ 43″ W. Isolated from roots and soil of wheat *Triticum aestivum* in agriculture field, 16 August 2000. *T. Paulitz* 90089 (HOLOTYPE: DAOM 230114. ISOTYPE: CBS 110198. Living culture ex type ATCC MYA 2560); Harrington, Lincoln Co., Lat: 47° 23′ 54″ N, Long. 118° 09′ 37″ W. Isolated from

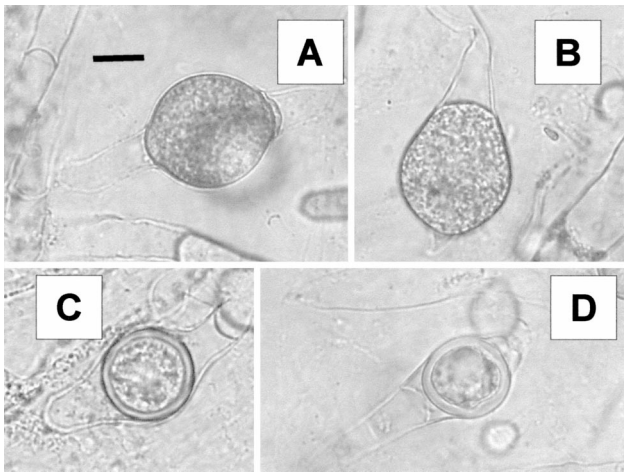

FIG. 6. Sporangia (A, B) and oospores (C, D) of *Pythium abappressorium*, formed within appressoria. Bar represents 10 μm.

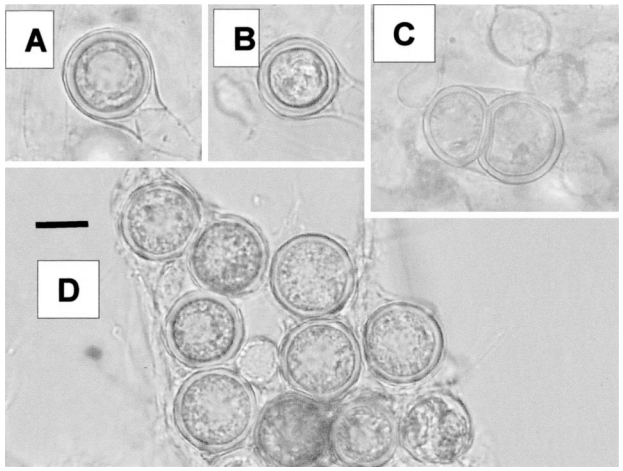

FIG. 8. Plerotic oospores of *Pythium abappressorium*. Antheridia are occasionally hypogynous (A, B), and occasionally, two oospores will be formed in one oogonium (C). Bar represents 10 μm.

roots and soil of wheat *Triticum aestivum* in agriculture field, 4 August 2000. *T. Paulitz* 020125. DAOM 230112, CBS 110196. ATCC MYA 2561; Dixie, Walla Walla Co. Lat: 46° 07′ 35″ N, Long. 118° 03′ 56″ W. Isolated from roots and soil of wheat *Triticum aestivum* in agriculture field, 1 August 2000. *T. Paulitz* 020135. DAOM 230113, CBS 110197. ATCC MYA 2562.

Etymology. Ab, Latin = away from, departing from, as in *ab*normal + appressorium.

DISCUSSION

Pythium abappressorium has several unique morphological characters not shared by other *Pythium* spp. Many species, such as *P. arrhenomanes* Drechsler, *P. sylvaticum* Campbell and Hendrix and *P. graminicola* Subramanian form appressoria. Appressoria are formed in contact with solid surfaces, such as cellophane membranes, petri dish surfaces, or grass leaves, but are rarely formed in agar media. Only one species has recently been shown to form reproductive structures from the appressoria, *P. contiguanum* Paul (syn. *P. dreschleri* Paul) (Paul 2000). He showed both antheridia and oogonia arising from appressoria, but they did not appear to be formed within the appressorium, unlike *P. abappressorium*. Sporangia were not formed within the appressoria of *P. contiguanum*, which forms filamentous, inflated sporangia, unlike *P. abappressorium*. In *P. abappressorium*, sporangia, oospores, and appressorial swellings are formed from appressoria, usually intercalary. At maturity, the tapered remnants of the appressoria often remain attached. Sporangia and oospores can also be formed without appressoria. Another unique character was the small swellings produced within the appressoria. These are probably analogous to hyphal swellings. Unlike chlamydospores, these structures are thin-walled. It appeared that the cytoplasm retracted within the appressorium, and a septum was often formed between the appressorial swelling and the empty appressorium. Appressorial swellings may function as propagules, but this would have to be confirmed by observation of germination. For example, hyphal swellings of *P. ultimum* can survive in soil for long periods of time, and function as inoculum (Stanghellini and Hancock 1971). Another uncommon characteristic of the appressorium of *P. abappressorium* is the branching and clustering of the appressoria. In most species, appressoria are single or in chains. In *P. abappressorium*, a number of appressoria can form from a single stalk, as in *P. irregulare* Buisman (Agnihotrti 1969). *Pythium myriotylum* Drechsler also forms clustered appressoria.

Oospores are mostly plerotic and antheridia are mostly monoclinous and paragynous, although some diclinous and hypogynous antheridia were seen. Not all isolates produced oospores in grass leaves, but since single hyphal-tipped cultures produced oospores, this species is homothallic.

This species was isolated as part of a comprehensive survey of wheat and barley fields in eastern Washington State. *Pythium* species were identified with both classical and molecular techniques, namely sequencing of the ITS region. This latter technique allows the identification of species that are difficult to distinguish from one another (e.g., *P. ultimum* vs *P. debaryanum*), species that do not form oospores, or species that have not been described. Sprague (1946) reported nine species of *Pythium* from grasses and grains in the northern Great Plains and western states. These included *P. aristosporum* Vanterpool, *P. arrhenomanes* Drechs., *P. ultimum*, *P. irregulare* Buisman, *P. debaryanum* Auct. non R. Hesse, *P. monospermum* Pringsh., *P. periilum* Drechs., *P. rostratum* Butler, and *P. tarticrescens* Vanterpool. Most of Sprague's (1946) references were to isolates from the northern Great Plains, but *P. ultimum*, *P. rostratum* and *P. tardicrescens* were specifically mentioned as having been found in the Pullman, Washington area. Chamswarng and Cook (1985) identified ten species from both wheat roots and wheat field soils near Pullman Washington, including *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, *P. irregulare*, *P. torulosum* Coker & Patterson, *P. volutum* Vanterpool, and *P. heterothallicum* Campbell & Hendrix. They also described two unidentified species, *Pythium* sp. ''E'' and ''D''. Neither resembled *P. abappressorium*. *Pythium* sp. ''E'' formed much larger sporangia (30 μm × 24 μm diameter) that were often catenulate. Oospores were only formed when crossed with other isolates. *Pythium* sp. ''D'' exhibited a sharp chrysanthemum pattern in culture, and grew at one-half the growth rate of *P. abappressorium* at 20 C. It is unclear why *P. abappressorium* was not detected in the work of Chamswarng and Cook (1985). They isolated directly from wheat roots or used soil dilutions. In our experience, fewer species are isolated directly from wheat roots, possibly because diseased roots are rotted away and not recovered on the root washing sieves. Chamswarng and Cook (1985) also planted wheat seeds into the soils, and isolated from the roots of the seedlings. Both methods probably favor highly virulent species of *Pythium*. Using grass leaves in a primary screening may favor more weakly pathogenic species. Another possibility is misidentification. If isolated strains did not produce oospores, they could have been misidentified as *P. ultimum* var. *ultimum*, which produces hyphal swellings of similar size. *Pythium abappressorium* appears to be widespread in eastern Washington and was found at over 50% of the

locations surveyed (Paulitz unpubl). Out of 230 randomly picked isolates that were sequenced, 62 or 27% were *P. abappressorium*.

This species was also found independently two years earlier by Mazzola et al (2002), being recovered from the roots of apple in three of six orchards surveyed in central Washington State. Isolates were obtained from the CV orchard in Orondo, the DO orchard in Zillah and the GC orchard in Manson, Washington. The CV and GC orchards have been under continuous orchard management for over 60 yr, while the DO orchard was established in 1994. Prior to orchard establishment, the CV and GC orchard sites were native shrub-steppe vegetation and the DO orchard had been in long-term perennial pasture. This species typically constituted a minor component of the total *Pythium* population recovered from the roots of apple at these sites, but was the dominant non-pathogenic species recovered from the roots of apple at the DO and GC orchards. Some of these isolates (DAOM Numbers 229185, 229186, 229191, 229192, 229193, 229194) were sequenced by C. A. Lévesque, and matched the *P. abappressorium* isolates of Paulitz reported here (Lévesque, pers comm).

P. abappressorium is not pathogenic to apples, but can cause damping-off and embryo infection on wheat. In tests in pasteurized soil at 16 C, an inoculum density of 1000 cfu/g resulted in significant damping-off of spring wheat. *Pythium* spp. can be found in PNW soils in populations ranging from 100 to 1000 cfu/g (Cook et al 1987). However, pasteurized soil may increase the inoculum potential of *P. abappressorium*, compared to natural soil. In tests in natural soil at similar inoculum densities, (Paulitz unpubl), we did not observe a significant reduction in emergence, but did observe symptoms of embryo infection, including first leaves that were smaller and twisted. In natural soil, *P. ultimum* and *P. irregulare* appeared to be more virulent than *P. abappressorium*. More detailed studies are ongoing to compare the virulence of *P. abappressorium* to other *Pythium* species.

In culture, *P. abappressorium* is fast growing, with a similar growth rate to *P. ultimum*. It has a broad temperature optimum, with similar growth rates over the range of 15–30 C. No growth was detected at 35 C, but this fungus was capable of slower growth at 5 and 10 C. Soil temperatures in 1998 at Pullman, Washington at 5 cm under conventional tillage averaged 8.8, 12.2, 14.5, 20.2, 23.8, 8.3, and 4.2 C during April, May, June, July, August, October, and November, respectively (D. Huggins, unpubl). This indicates that *P. abappressorium* can be active during the early fall and spring, when winter and spring wheat are planted in the Pacific Northwest, and when root establishment would take place.

The ITS1 DNA sequences of *P. abappressorium* were compared with over 1200 sequences from a worldwide collection that included types and neotypes of most species of *Pythium* (Lévesque unpubl), using BLAST. There were no matches to any of these isolates. This is further confirmatory evidence that *P. abappressorium* has not been described before. *P. macrosporum* had the closest sequence, but it had less than 80% homology with the ITS1 region of *P. abappressorium*, and is quite different morphologically. In general, the ITS1 sequences of *Pythium* isolates within a species may vary by only 1 or 2 base pairs.

In conclusion, a new species of *Pythium* has been described from Washington State. This species was widely distributed in wheat and barley fields, and was pathogenic on wheat. It was also recovered from soil and apple roots from various orchard sites throughout the primary apple production region of central Washington, ranging from Manson in the north to Zillah in the south. *P. abappressorium* was highly competitive in the rhizosphere of apple and was able to suppress colonization by pathogenic species of *Pythium*, including *P. sylvaticum* and *P. ultimum*, resulting in enhanced apple growth. Thus, *P. abappressorium* has potential to serve as a biological agent for control of these root pathogens of apple. The interactions between *P. abappressorium* and other *Pythium* species in Washington soils remain to be studied.

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